EXHIBIT 1

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Increased Heterologous Protein Production in *Aspergillus niger* Fermentation through Extracellular Proteases Inhibition by Pelleted Growth

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The dependence of filamentous fungal protease secretion on morphology was investigated by employing the recombinant *Aspergillus niger* strain AB4.1[pgpdAGLAGFP] which contains a gene for the glucoamylase-GFP (green fluorescence protein) fusion protein. Different inoculum levels were used to obtain different sizes of pellet or free mycelia. The extracellular protease activity of the cultures varied with the pellet size and decreased dramatically when the morphology was changed from free mycelia to pellets. The culture with an optimal pellet size of 1.8 mm was obtained from an inoculum of 4×10^5 spores/mL. It resulted in a specific protease activity of 158 units/L, only one-third of that in free mycelial growth, and a maximum specific GFP yield of 0.98 mg/g (cell mass) compared to 0.29 mg/g for free mycelial growth with an inoculum of 10^7 spores/mL. The results indicate that this bioprocessing strategy can be effectively used to inhibit protease activity in filamentous fungal fermentation and thereby to enhance heterologous protein production.

Introduction

Proteolytic degradation by fungal proteases is recognized as one of the major problems interfering with efficient heterologous protein production in the fungal fermentation industry (*1, 2*). Current strategies are focused on selecting protease-deficient mutants (*3–5*). Little has been reported on suppressing fungal protease secretion by bioprocess engineering means such as cell immobilization, fed-batch culture, pH control, and morphology control. In our previous work (*6*), inhibition of extracellular protease secretion by cell immobilization was observed. The maximum specific activity of the protease secreted from the immobilized cells of wild-type *Aspergillus niger* was reduced to 25% of that from free filamentous culture in shake flasks, demonstrating a high potential of bioprocess engineering strategies in fungal protease inhibition.

It is well-known that filamentous fungal cells exhibit two extreme types of morphology in submerged cultures: pelleted and free filamentous forms. The latter is common in industrial fermentation. However, reduced extracellular protease secretion was found in pelleted growth in our laboratory, which is beneficial for heterologous protein production. However, pelleted growth may result in reduced cell mass as a result of substrate limitation in the dense core of the pellet when the pellets exceed a "critical radius" (*7, 8*). Therefore, the ability to obtain and control a certain pellet size is important. Parameters influencing pellet formation include inoculum level (*9*), initial pH (*10*), agitation (*8, 11*), medium composition (*8, 12*), polymer additives (*13, 14*), and surface-active agents (*15*). Among them, the inoculum level is regarded as the most important in determining the pellet size developed (*16*).

In this work, a recombinant *A. niger* strain containing a glucoamylase-GFP (green fluorescence protein) fusion protein gene was employed as a model system to investigate the relationship between extracellular protease secretion and fungal morphology. GFP, a heterologous protein for *A. niger*, is widely used as a fluorescent reporter protein in bioprocess development (*17*). When the GFP gene is fused with that of glucoamylase, a protein efficiently secreted by *A. niger*, the resulting GFP–glucoamylase fusion protein is also secreted efficiently by *A. niger*. The fusion protein is cleaved in the broth after secretion. To obtain an optimal pellet size for reduced protease activity and enhanced GFP production, control of inoculum level was investigated in this work.

Materials and Methods

Fungal Strain and Medium. The recombinant *A. niger* strain AB4.1[pgpdAGLAGFP], which carries the glucoamylase-GFP fusion protein gene, was kindly provided by Dr. P. J. Punt of the TNO Nutrition and Food Research Institute, The Netherlands. The strain AB4.1 is a *pyrG1* derivative of N402 (*18*), and N402 is a *cspA1* derivative of strain ATCC 9029. The GFP gene is fused with the region encoding amino acid 1-514 (G2 form) of *A. niger* glucoamylase in order to increase the secretion efficiency of GFP after expression.

Culture Conditions. The recombinant *A. niger* was grown on YM medium containing 3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone, and 10 g/L dextrose. Cultures were grown in 250 mL shake flasks containing 100 mL of medium. Spores for inoculation were obtained by adding 20 mL of sterilized water to 5-day-old slants. The spore number in suspension was counted using a hemocytometer before inoculation. Different volumes of spore solution were added to the culture medium to give a desired inoculum level. The flasks were then placed in an Innova 4000 shaker (New Brunswick) at 24 °C and

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200 rpm for 6 days before harvesting. The experiments were conducted in duplicates. All reported values are averages of the duplicate trials.

Analytical Procedures. The samples drawn from each flask were filtered. The filtrate was collected for measurements of sugar content, protease activity and GFP concentration, respectively. The biomass was washed three times with deionized water and then dried in an oven at 70 °C for 24 h for the determination of dry weight.

Sugar content was analyzed enzymatically by a glucose kit (Sigma, catalog no. 315-100). Extracellular protease activity was determined according to the method of van den Hombergh et al. (*6*). A 450 μL sample was incubated with 50 μL of 1% (w/v) BSA (fraction V, Sigma Chemicals) in 0.1 M sodium acetate buffer (pH 4.0) at 37 °C. At 30, 60, and 90 min, the reactions were terminated with 500 μL of 10% (w/v) trichloroacetic acid (TCA). After incubation at 0 °C for 0.5 h, the precipitated proteins were removed by centrifugation at 9000 rpm for 5 min, and the optical density of the TCA-soluble fraction was measured at 280 nm. One unit (U) of protease activity was defined as a change of one absorbance unit per h at 280 nm for 1 mL of reaction precipitation mixture as described above. Extracellular protease activity was expressed as U/L.

Extracellular GFP was assayed with an HP 1000 series fluorescence detector (Hewlett-Packard) using the software package ChemStation (Hewlett-Packard). The conditions for fluorescence measurements were as follows: excitation at 488 nm, emission at 520 nm, and temperature 25 °C. A 0.2 M phosphate buffer (pH 7) was pumped continuously into the flow cell of the fluorescence detector through an injector. A 50 μL culture medium was injected each time for the measurement of relative fluorescence units (RFU). Pure GFP (Clontech, Palo Alto, CA) was used for calibration.

For the determination of pellet size, the pellets harvested from each culture were divided into 6 groups: 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 mm. The number of pellets in each group was counted from at least 200 randomly chosen pellets from each flask. The average diameter (D) was calculated as

$$D = \sum_{i=1}^{6} E_i d_i$$

where E_i is the percentage of pellet number in each size group and d_i = 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 mm (*19*).

Results and Discussion

Fungal Protease Secretion and GFP Degradation. The time course for protease secretion in free filamentous cell culture of the *A. niger* is shown in Figure 1. Extracellular protease secretion started after the cell growth approached the stationary phase around the end of day 2 when the glucose in the medium was almost depleted. The maximum protease activity detected at the end of the culture was 2850 U/L. In the filamentous cell culture above, only trace amount of GFP (1.3 mg/L) was detected at the end of day 6.

To test the degradation of GFP by proteases, the 6-day-old culture broth was collected by vacuum filtration. The cell-free broth (100 mL) was spiked to a concentration of 10 mg/L GFP with standard GFP and put in the Innova shaker at 24 °C and 200 rpm for 2 days. The protease activity and GFP concentration were measured at 6 h intervals as shown in Figure 2. The protease activity in the culture broth decreased only slightly after 2 days.

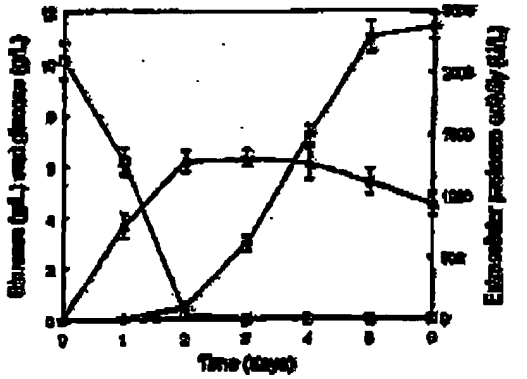

Figure 1. Time course for cell growth and extracellular protease secretion in recombinant *A. niger* culture. Inoculum: 2.0×10^5 spores/mL. Biomass (▲), sugar content (□), and protease activity (●).

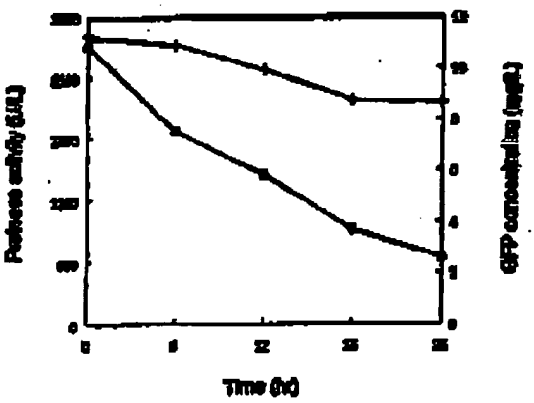

Figure 2. Degradation of GFP by fungal protease. Protease activity (●) and GFP concentration (■).

GFP concentration declined dramatically from 10.8 to 2.6 mg/L (Figure 2), indicating the degradation of GFP by proteases. It is possible that some other factors such as photobleaching and oxidation/reduction may also be responsible for the reduction of GFP concentration in the solution. However, a control experiment using the fresh YM medium (protease-free) to incubate with 10 mg/L standard GFP under the same conditions revealed that GFP concentration was only 8.5% lower after 2 days. Thus, protease degradation was likely the main factor contributing to the GFP loss.

Relationship Between Protease Activity and Pellet Size. To compare the extracellular protease activity and GFP production among the cultures with different fungal morphological states, five inoculum levels, 10^3, 10^4, 10^5, 10^6 and 10^7 spores/mL, were applied to the cultures. On the basis of an investigation of fermentation kinetics (data not shown here), all of the cultures reached maximum biomass before the end of the fourth day, and the protease secretion approached the highest by the end of the sixth day (2 days later). Fungal spores usually germinated within 10 h of inoculation and then grew very fast within the initial 2 days. GFP, a primary metabolite, was produced and secreted during cell growth. In this case, the difference in the profile of cell growth and GFP secretion resulting from varying inoculum levels can be

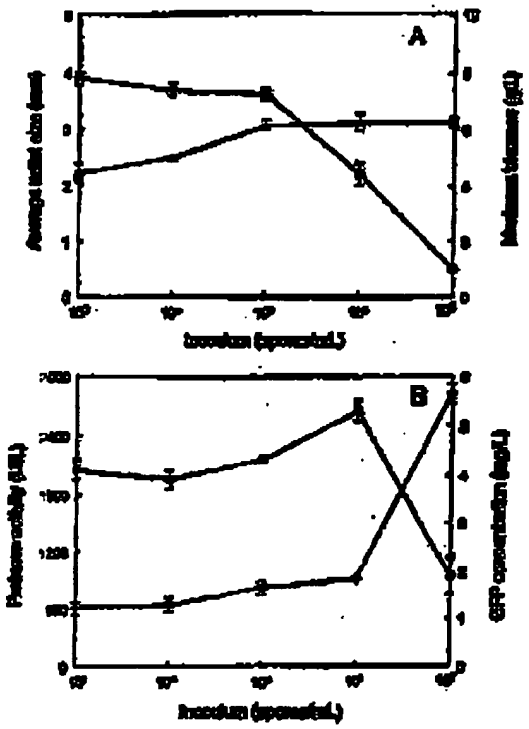

Figure 3. Dependence of pellet size (○), biomass (△), protease activity (+), and GFP yield (▽) on inoculum levels in recombinant *A. niger* cultures. The maximum biomass and pellet sizes were determined at the end of day 3 after inoculation; protease activity and GFP were measured at the end of day 6 after inoculation.

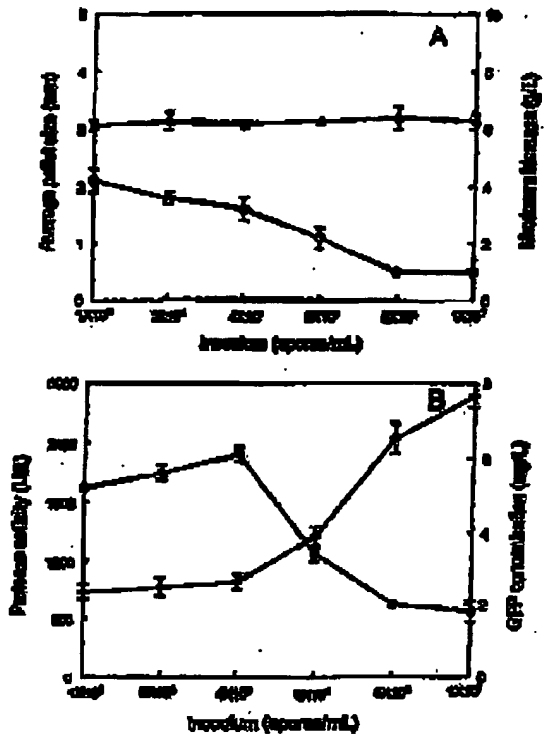

Figure 4. Dependence of pellet size (○), biomass (△), protease activity (+), and GFP yield (▽) on inoculum levels in recombinant *A. niger* cultures. The maximum biomass and pellet sizes were determined at the end of day 3 after inoculation; protease activity and GFP concentration were measured at the end of day 6 after inoculation.

ignored when the cultures were harvested at the end of the sixth day.

The resulting pellet size, maximum biomass, protease activity, and GFP yield of each culture are shown in Figure 3. The size of pellets in each culture depended on its inoculum level. Increased inoculum levels resulted in reduced pellet sizes. A sharp reduction in pellet size from 3.6 to 0.8 mm was observed when the inoculum level increased from 10^5 to 10^7 spores/mL (Figure 3A). The culture resulting from the inoculum level of 10^7 spores/mL consisted of free mycelia instead of pellets. Lower inoculum levels in this work resulted in pelleted growth. Figure 3A shows that, corresponding to the decrease in pellet size, the biomass increased from 4.8 to 6.2 g/L, indicating the influence of substrate mass transfer limitation on cell growth.

Figure 3B reveals that extracellular protease activity increased with the inoculum levels or the reduced pellet size. The protease activity increased dramatically from 820 to 2550 U/L when the inoculum level was raised from 10^5 to 10^7 spores/mL or the morphology was changed from pellet growth to free mycelial growth, demonstrating the inhibitory effect of pelleted growth on extracellular protease secretion. GFP production exhibited a peak of 5.3 mg/L at 10^5 spores/L inoculum level, and then the value dropped sharply to 1.9 mg/L at 10^7 spores/L inoculum level when pelleted growth was switched to free filamentous growth. Because the maximum biomass of these cultures are different, it is more reasonable to compare the specific yields (based on the corresponding maximum biomass) of protease and GFP among these cultures. The results are shown in Table 1. The specific protease activity was reduced by more than 3-fold in pelleted growth compared to filamentous growth, while the maximum specific GFP production reached 0.98 mg/g, 2.7 times greater than that in filamentous growth.

Table 1. Specific Yields of Extracellular Proteases and GFP using *A. niger* Cultures with Five Different Inoculum Levels

	inoculum (spores/mL)				
	10^3	10^4	10^5	10^6	10^7
average pellet size (mm)	3.6	3.7	3.6	2.3	0.8
specific protease activity (U/g)	120	122	154	148	455
specific GFP production (mg/g)	0.62	0.76	0.91	0.89	0.91

Figure 3B shows a sharp increase in protease level between inoculum levels of 10^5 and 10^7 spores/mL. Further investigation was carried out with inoculum ranging from 10^5 to 10^7 spores/mL in order to find a more precise optimal inoculum. As shown in Figure 4A, the maximum biomass of each culture remained in a narrow range of 6.1 to 6.3 g/L while the pellet size declined from 3.1 to 0.8 mm with an increase in inoculum level from 10^5 to 10^7 spores/mL. In Figure 4B, a maximum GFP concentration of 5.3 mg/L was observed at the inoculum level of 4×10^5 spores/mL (average pellet size, 1.9 mm). The GFP concentration decreased rapidly following the sharp increase in protease activity when the inoculum level was raised. Therefore, the pellet size of 1.5 mm, which resulted from 4×10^5 spores/mL inoculum level, could be regarded as optimal in view of protease activity

Table 2. Specific Yields (Based on Corresponding Maximum Biomass) of Extracellular Protease and GFP in Recombinant *A. niger* Cultures with Inoculum Levels Ranging From 10^5 to 10^7 Spores/mL

	Inoculum (spores/mL)					
	1×10^5	2×10^5	4×10^5	6×10^5	8×10^5	1×10^7
average pellet size (mm)	2.1	1.8	1.5	1.1	0.8	0.5
specific protease activity (U/g)	144	147	159	231	393	469
specific GFP production (mg/g)	0.84	0.98	0.98	0.65	0.51	0.29

Table 3. Comparison of Pelleted Growth with Free Mycelia Growth

inoculum (spores/mL)	growth type	maximum biomass (g/L)	average specific growth rate (hr^{-1})	Y_{xs}[a]	specific protease activity (U/g)	specific GFP production (mg/g)
3.0×10^7	free mycelia	8.3	0.048	0.65	988	0.2
4.0×10^5	pellets	6.1	0.054	0.60	184	0.98

[a] Y_{xs} is the yield coefficient in g dry weight/g glucose.

and GFP production. Under this inoculum level, the extracellular protease activity detected was 980 U/L, much less than the 2870 U/L detected in filamentous growth with an increased inoculum. The specific yields of extracellular protease and GFP of these cultures are shown in Table 2.

The pelleted growth at inoculum level of 4×10^5 spores/mL resulted in a maximum specific GFP yield of 0.98 mg/g. The specific protease activity in this culture was as low as one-third of that determined in free mycelial growth. The enhanced GFP production may be a result of the change of morphological state from free mycelia to pellets. However, GFP as a heterologous protein to *A. niger* has no evident physiological functions to fungal metabolism. Its biosynthesis may not be significantly affected by the morphological change. The research of Johnson et al. (22) with heterologous protein production in *Aspergillus awamori* cultures confirmed that the morphological differences between pellets and free mycelia had only a limited effect on product formation. Therefore, the reduced extracellular protease secretion is likely a leading factor in the increased GFP production. Moreover, even though fungus grew as pellets with low extracellular protease secretion, the GFP production varied with the pellet sizes. Reduced GFP production was observed when the pellets became as large as 4 mm, as shown in Figure 3B, which may be related to the decreased metabolic activity of the cells caused by mass transfer limitation in larger pellets. In Figure 3A, with the increase in pellet size, maximum biomass declined, confirming that reduced metabolic activity occurred in larger pellets. However, when the pellets were developed with the sizes less than the "critical radius" (about 2 mm) (7, 8), cell growth was not significantly affected by mass transfer limitation, because the oxygen concentration remained greater than zero inside the whole pellets. This explains why the maximum biomass accumulated was almost the same among the cultures with pellet size ranging from 0.5 to 2.1 mm (Figure 4A), but the GFP production still seems affected by mass transfer limitation. Its maximum value occurred corresponding to the pellet size of 1.5 mm.

The mechanism by which pellet formation reduces the protease secretion has not yet been elucidated. It is generally accepted that the proteases are produced in response to nutrient limitation or adverse microenvironmental conditions (21). Wiebe (23) suggested that secretion of hydrolytic and other enzymes into the medium by organisms could be a stress response to the culture conditions, thus limiting of protease activity by altering the physiological state of the cells is possible. More specifically, oxygen is regarded as the main factor influencing the protease production (25, 26). Kole et al. (25) claimed that when *Bacillus* cells were exposed to high oxygen concentration, more biomass and higher protease activity were obtained. Moon and Parulekar (26) also concluded that the synthesis of extracellular proteases was repressed under oxygen limitation. In this work, formation of fungal pellets reduced the hydrodynamic stress to the cells. In the meantime, reduced oxygen concentration occurred inside the pellets as a result of mass transfer limitation, and as a result, extracellular secretion of proteases was suppressed to some extent.

In addition to the reduced protease secretion by pelleted growth, some technical problems encountered in free mycelial cultures, such as increased wall growth and reduced mixing efficiency and oxygen transfer due to the high viscosity of the broth, could be solved by growing the filamentous organisms in the form of pellets (16). The major consequences hereof are the lower medium viscosity compared to growth as free mycelia and the occurrence of intrapellet nutrient concentration gradients. Cultivation in the form of pellets also improves harvesting through eased filtration of the medium (27). Therefore, the problem here comes to the minimization of the intracellular mass transfer limitation within the pellets. By controlling the formation of pellets to an appropriate size, usually less than 2 mm, the intracellular mass transfer limitation will be largely prevented (8).

Characterization of Fungal Pellet Growth. The time course for the *A. niger* pelleted growth and extracellular protease secretion at 4×10^5 spores/mL is shown in Figure 5. The profiles of cell growth and extracellular protease secretion in Figure 5 are similar to those shown in Figure 1 for filamentous growth, except that the pellets did not grow as fast as free mycelia for the first day of culture. The biomass accumulated by pelleted growth on day 1 was only 40% of that by free mycelia growth. The extracellular protease increased sharply in both cases when the cell growth entered the stationary phase. Table 3 shows a comparative summary of the two cultures.

To some extent, pelleted growth may be regarded as the self-immobilization culture of fungal cells that eliminates the need for artificial immobilization supports but has the same advantages as the artificially immobilized cell cultures including low liquid viscosity and better mixing. The success of this approach relies on the formation of pellets of regular shape and size. It was observed that all of the pellets developed in these cultures appeared as spherical aggregates with different sizes. The pellet size distribution of the culture with inoculum of 4×10^5 spores/mL is illustrated in Figure 6.

The size of pellets mainly concentrated within the range of 1–3 mm, which accounted for 90% of the total pellet number. Only a small percentage (2%) of pellets

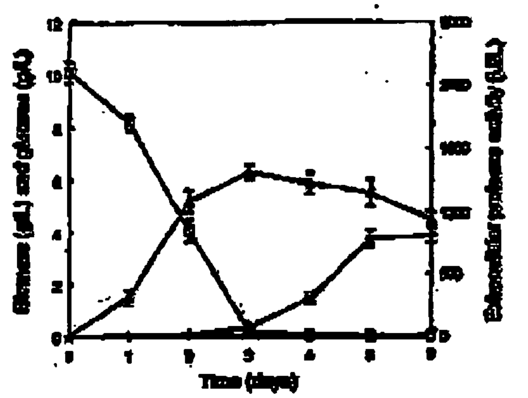

Figure 5. Time course for cell growth and extracellular protease secretion in recombinant *A. niger* culture. Inoculum: 4 × 10⁵ spores/mL. Biomass (▲), sugar content (□), and protease activity (▼).

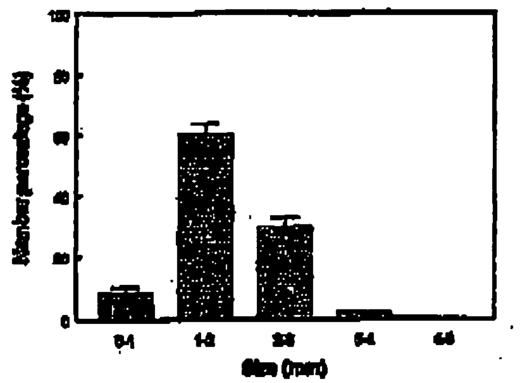

Figure 6. The distribution of pellet size of recombinant *A. niger* cultures with inoculum level of 4 × 10⁵ spores/mL.

was found with diameters greater than 3 mm. The culture broth remained almost clear throughout the culture since there were hardly any dispersed hyphae in the broth. Such a fungal broth is highly desirable in industrial fermentation.

The research described above was carried out in shake flasks. The conditions for pellet size control should be modified when applied to bioreactors, in particular when the effect of agitation is considered. However, reduced protease production is still predicted with pellet growth as compared to free mycelial growth.

Conclusions

In *A. niger* cultures, extracellular protease secretion was related to the morphological state. A dramatic decrease in protease activity was found when the fungal cells grew as pellets instead of free mycelia. The inoculum level was found to be directly related to morphology. An optimal inoculum level of 4 × 10⁵ spores/mL in this work resulted in a culture consisting of a pellet size of 1.6 mm, which produced a specific protease activity of 158 U/L and a specific GFP yield of 0.88 mg/g, much higher than the 0.59 mg/g produced in filamentous growth.

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